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LSD1 cooperates with CTIP2 to promote HIV-1 transcriptional silencing

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ABSTRACT

Microglial cells are the main HIV-1 targets in the central nervous system (CNS) and constitute an important reservoir of latently infected cells. Establishment and persistence of these reservoirs rely on the chromatin structure of the integrated proviruses. We have previously demonstrated that the cellular cofactor CTIP2 forces heterochromatin formation and HIV-1 gene silencing by recruiting HDAC and HMT activities at the integrated viral promoter. In the present work, we report that the histone demethylase LSD1 represses HIV-1 transcription and viral expression in a synergistic manner with CTIP2. We show that recruitment of LSD1 at the HIV-1 proximal promoter is associated with both H3K4me3 and H3K9me3 epigenetic marks. Finally, our data suggest that LSD1-induced H3K4 trimethylation is linked to hSET1 recruitment at the integrated provirus.

INTRODUCTION

Eukaryotic DNA is wrapped around core histone proteins to form the chromatin (1). It is now well-established that the local state of chromatin influences transcription. A heterochromatin environment is more compact and structured than euchromatin, and is therefore repressive for transcription. On the contrary, euchromatin, a relaxed state of chromatin, is associated with active transcription. The compaction of chromatin and its permissivity for transcription depend on post-translational modifications of histones such as acetylation, methylation, sumoylation, phosphorylation and ubiquitinylation (2). It has been proposed that combination of such different covalent modifications of histone proteins may constitute a histone code and could be used to determine transcriptional status (3,4). The acetylation of a lysine in histones is mainly linked to gene activation, while lysine methylation can be associated to both gene activation and repression (5). For instance, methylation of H3K4 (Histone 3 Lysine 4), H3K36 and H3K79 have been associated to gene activation, whereas methylation of H3K9 and H3K27 have been linked to gene repression (6). The transcriptional activity of a gene is also regulated by the degree of histone methylation (mono, di or trimethylation). Trimethylation of H3K4 (H3K4me3) can exist in conjunction with H3K9 acetylation and is correlated to the activation of transcription (7,8), whereas dimethylation of H3K9 is linked to the recruitment of the deacetylase complex Set3, which induces gene repression (9). However, this epigenetic code is not always correlated with a corresponding transcriptional activity (10,11).

To date a great number of methyltransferases and demethylases has been shown to shape the pattern of lysine methylation. SUV39H1 has been involved in heterochromatin formation at the HIV-1 promoter and, as a consequence, in HIV silencing (12,13). The lysine specific demethylase (LSD1), discovered in 2004 (14), was initially associated to gene repression (15,16). This enzyme, which removes methyl groups from mono and dimethylated

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H3K4, was characterized as a REST co-repressor. Additional binding partners of the LSD1-CoREST complex are histone deacetylases HDAC1 and HDAC2, which have been linked to transcriptional repression of several genes including the HIV-1 provirus (17). However, LSD1 has also been involved in the activation of transcription (18). Indeed, Metzger *et al*. (18) showed that LSD1 and the androgen receptor co-localize on promoters following hormone treatment. The recruitment of these two proteins did not alter H3K4 methylation but stimulated H3K9 demethylation, which led to transcriptional activation. Since LSD1 cannot remove methyl groups from trimethylated lysines, it has been proposed that LSD1 could serve as an anchored protein to recruit directly or indirectly H3K9 specific histone demethylases. Furthermore, both LSD1 and the H3K9 demethylase of the Jumonji-containing class belong to the same chromatin-remodelling complex, further supporting this hypothesis (19). However, the discovery that inhibition of LSD1 prevents lytic replication of the herpes simplex virus (HSV) as well as its reactivation from latency has added another level of complexity in our understanding of LSD1 function in gene regulation. Indeed, it was shown that HCF-1, which is a component of the SET1 and MLL1 H3K4 methyltransferase complexes, recruits LSD1 and induces H3K4 trimethylation and transcriptional activation of the HSV promoter (20–23). From an elegant approach that uses a variation of genome-wide chromatin immunoprecipitation called chromatin-immunoprecipitation (ChIP)-DSL, it appeared that LSD1 plays an even broader role in transcriptional activation as 80% of the 4200 LSD1-positive promoters were associated with RNA polymerase II and gene activation (24). These results underlined the dual role of LSD1 in gene activation and repression, and highlighted the complex role of lysine methylation in epigenetic regulation.

Here, we focused on the molecular mechanisms underlying HIV-1 transcription. We studied in more details the molecular mechanisms involved in the establishment and maintenance of HIV-1 latency in microglial cells, the main HIV-1 target cells in the central nervous system (CNS) (25). These long-lived latent reservoirs constitute a major obstacle to the eradication of HIV-1. Understanding the cell-type specific molecular mechanisms of establishment, maintenance and reactivation of HIV-1 latency is therefore crucial to achieve an efficient therapeutic intervention, in which the ultimate goal is to completely eradicate both latently and productively infected cells (26,27). We have previously shown that COUP-TF interacting protein 2 (CTIP2), a recently cloned transcriptional repressor that can associate with members of the COUP-TF family (28), inhibits HIV-1 replication in human microglial cells (29,30) by recruiting a chromatin-modifying complex (13). Indeed, our work showed a concomitant recruitment of histone deacetylases HDAC1 and HDAC2, and methyltransferase SUV39H1 to the viral promoter by CTIP2. Heterochromatin formation at the HIV-1 promoter has been linked to post-integration latency (26,31), suggesting that transcriptional repressors such as CTIP2 are involved in the establishment and maintenance of viral persistence and post-integration latency in the brain. The co-repressor CTIP2 has an even more pleiotropic action by regulating the expression of host genes in the infected cell. In this context, we have shown that CTIP2 silences p21 gene transcription by inducing epigenetic modifications, such as deacetylation and methylation of histones (32). This effect may indirectly favour HIV-1 latency since activation of the p21 gene stimulates viral gene transcription in macrophages (33). Moreover, CTIP2 counteracts HIV-1 Vpr which is required for p21 expression. In a recent article, we suggested that all these factors together contribute to HIV-1 transcriptional latency in microglial cells (32).

In the present work, we show that LSD1 represses HIV-1 transcription and viral expression in a synergistic manner with CTIP2. We report for the first time that recruitment of LSD1 at the HIV-1 proximal promoter is associated with both H3K4me3 and H3K9me3 epigenetic marks.

MATERIAL AND METHODS

Plasmids

Most of the constructs used in our assays have been described previously: pcDNA3, pFLAG-CTIP2 (28), pNL-4.3, pVSV.G, pRFP-CTIP2 (29), pTat-GFP (30), pshRNALSD1, pshRNA-Control and pFLAG-LSD1 were provided by E. Metzger and R. Schule (18). The episomal LTR-LUC, pshRNA-CTIP2, pSirenZsGreen-shRNA-CTIP2 plasmids have also been described (13).

Cell culture

The human microglial (provided by M. Tardieu, Paris, France) (34) and HEK 293 T cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 100 U/ml penicillin–streptomycin. CTIP2 knocked-down microglial cells expressing shRNA-CTIP2 were stably established by infection of microglial cells with a pSirenZsGreen-ShRNA-CTIP2-based retrovirus as described by the manufacturer (Clontech Lab. Inc.). CTIP2 shRNA-expressing cells were sorted by flow cytometry for the concomitant expression of the ZsGreen protein and cultured in DMEM. The CTIP2 knock-down efficiency was controlled by western blot and ChIP experiments. The monocytic HIV-1 infected U1 cell line was maintained in RPMI 1640-Glutamax I medium supplemented with 10% fetal bovine serum and 50 U/ml penicillin–streptomycin.

Co-immunoprecipitation assays

HEK 293 T cells cultured in 100-mm diameter dishes were transfected using the calcium phosphate co-precipitation method with the indicated pFLAG-CTIP2 (30 μg), pFLAG-LSD1 (30 μg), pSuper control or pcDNA3-FLAG control (30 μg) vectors. Two days post-transfection, immunoprecipitations were performed using the standard technique with M2 anti-FLAG (Sigma) overnight at 4°C. Finally, the immunoprecipitated complexes were processed for SDS–PAGE and western blot analysis.

SDS–PAGE and western blot analysis

SDS–PAGE was performed using standard techniques. Proteins were detected using antibodies directed against the FLAG epitope (M2 mouse monoclonal from Sigma), LSD1 (Abcam), CTIP2 (bethyl) and β-actin (Sigma). Proteins were visualized by chemiluminescence using the Super Signal Chemiluminescence Detection System (Pierce).

Luciferase assays

Microglial cells cultured in 48-well plates were transfected with the indicated vectors using the calcium phosphate co-precipitation method. Total amounts of DNA were normalized with the corresponding control vector. Two days later, cells were collected and luciferase activity was determined using the Dual-Glo™ Luciferase Assay System (Promega). Values correspond to an average of at least three independent experiments performed in triplicate.

Viral replication

Microglial cells cultured in 12-well plates were transfected using the calcium phosphate co-precipitation method with HIV-1 pNL-4.3 and the expression plasmids as indicated. Total amounts of DNA were normalized with the corresponding empty vector. HIV-1 replication was monitored as described previously (30). Values correspond to an average of at least three independent experiments carried out in triplicate.

Pseudotyped virion production and single-round infection

The plasmid pNL-4.3-Env was co-transfected with the envelope plasmid encoding the pVSV.G envelope protein into HEK 293 T cells. Virions were collected 48 h post-transfection. For single-round infection, microglial cells were incubated with the VSV-pseudotyped HIV-1 NL4.3-Env virus for 24 h at 37°C.

Chromatin immunoprecipitation assays

Microglial and CTIP2 knocked-down microglial cells cultured in 150-mm diameter dishes were subjected to single-round infection by the VSV-pseudotyped viruses 24 h before being processed for ChIP experiments. HEK 293 T cells cultured in 100-mm diameter dishes were transfected using the calcium phosphate co-precipitation method with the indicated vectors or the corresponding control vectors. ChIP assays were performed using the ChIP assay kit (Upstate) 48 h post-transfection. Microglial and U1 cells were mock-treated or treated with PMA (100 nM) for 24 h before ChIP assays. The primary antibodies used for ChIP were as follows: anti-LSD1 (Abcam), anti-CTIP2 (bethyl), anti-RNA Pol II (Santa Cruz), anti-Sp1 (Upstate), anti-H3pan (Upstate), anti-Ac-H3 (Upstate), anti-H3K9me3 (Upstate), antiH3K4me3 (Upstate), anti-WDR5 (Abcam) and anti-hSET1 (Abcam). Immunoprecipitated DNA was subjected to real-time PCR quantification. The amplified regions of the provirus are indicated in the legend section. The specificity of the enrichment has been controlled by amplifications of the GAPDH gene (13).

Indirect immunofluorescence and confocal microscopy

Microglial cells cultured in 48-well plates were transfected or not using the calcium phosphate co-precipitation method with the pFLAG-LSD1, pRFP-CTIP2 or pTat-GFP expression vectors. Cells were fixed and permeabilized as described previously (30). The cover slips were then incubated for 1 h at room temperature with primary antibodies directed against LSD1 (Abcam), hSET1 (Abcam) or against the FLAG epitope (M2 mouse monoclonal; Sigma). The primary immunocomplexes were revealed by CY3- or CY5-labeled secondary anti-species antibodies. The stained cells were analysed by confocal microscopy using a Zeiss laser scanning microscope (model 510 invert) equipped with a Planapo oil (63×) immersion lens (numerical aperture = 1.4).

RESULTS

LSD1 represses HIV-1 replication and transcription in microglial cells

The function of LSD1 in HIV-1 infected cells was investigated by using an LSD1 knock-down strategy. We co-transfected microglial cells with a complete HIV-1 infectious provirus (pNL-4.3) and with or without a shLDS1 expressing vector. The efficiency of the knock-down of LSD1 was checked by western blot (Figure 1A). As shown in Figure 1A, the knock-down of LSD1 was associated with a 6-fold increase in p24 production, which argues in favour of a repressive role of LSD1 in HIV-1 replication. We next investigated whether LSD1 has a direct impact on transcription of the HIV-1 genes since this protein is involved in the transcriptional regulation of many cellular genes. Microglial cells were transfected with the episomal LTR-Luc vector with or without the shLSD1 expressing vector in the absence (Figure 1B) or presence (Figure 1C) of Tat. In the absence of Tat, LSD1 repressed LTR transcriptional activity in a dose-dependent manner (Figure 1B columns 2 and 3). When Tat was expressed together with the shLDS1 vector, we observed a synergistic activation of LTR-driven transcription (Figure 1C column 4 compared to columns 2 and 3). Thus LSD1 inhibits HIV-1 replication as a result of transcriptional repression occurring at both the early Tat-independent and the late Tat-dependent steps.

LSD1-mediated repression of HIV-1 is associated with the epigenetic marks H3K4me3 and H3K9me3

To investigate whether LSD1 is recruited at the HIV-1 promoter *in vivo*, we performed ChIP assays using microglial cells transfected with the pNL-4.3 provirus. Over-expression of LSD1 was associated to an increase of H3K9 trimethylation (H3K9me3) and more surprisingly to an increase of H3K4 trimethylation (H3K4me3) (Figure 2A columns 3 and 4) in the proximal region of the HIV-1 promoter. In agreement, knocking-down endogenous LSD1 expression disfavoured H3K9 and

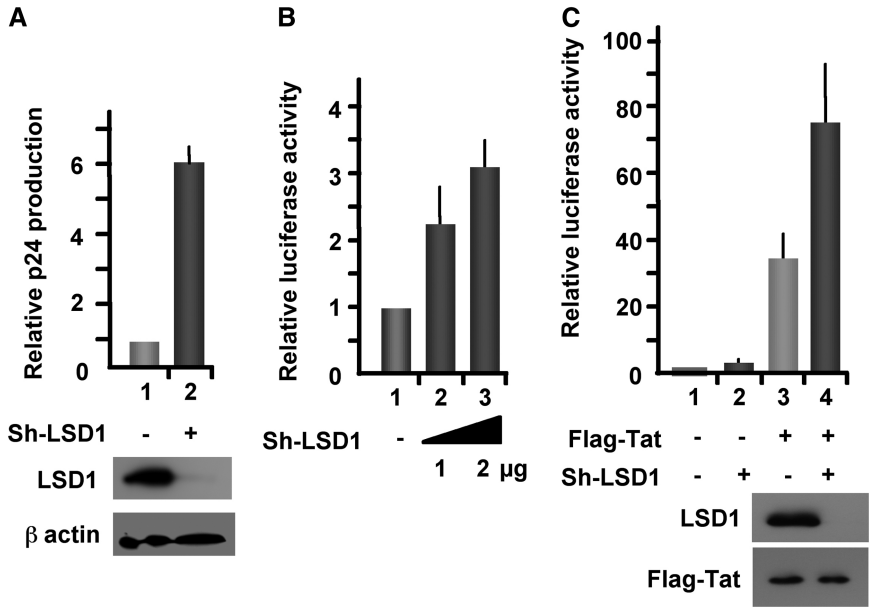

Figure 1. LSD1 represses HIV-1 gene transcription and viral replication. (**A**) Microglial cells were transfected with the pNL-4.3 and the indicated vectors. Culture supernatants were analysed for p24 Gag contents 48 h post-transfection. (**B** and **C**) Microglial cells were transfected with the episomal LTR-LUC and the indicated vectors. DNA amounts were normalized in all transfection assays with pshRNA-Control or pcDNA3-FLAG control vectors. Luciferase activities were measured 2 days post-transfection and expressed relative to the value obtained with episomal LTR-LUC alone. (A and C) The knock-down efficiency of sh-RNA constructs (versus sh-control) has been controlled by western blot analysis.

H3K4 trimethylation (Figure 2A columns 3 and 4). Interestingly, knocking-down LSD1 in HIV-1 transfected microglial cells was associated with a strong increase of H3 global acetylation level (Figure 2A column 6) and with a stable amount of H3 histones associated to the viral promoter (Figure 2A column 5). Furthermore, knocking-down LSD1 strongly increased the recruitment of the RNA pol II to the HIV-1 promoter, thereby confirming the activated status of the viral promoter (Figure 2A column 7). From these results we speculated that HIV-1 reactivation in latently infected U1 cells could be associated with a release of LSD1 and a concomitant decrease of H3K9 and H3K4 trimethylation levels at the HIV-1 promoter. To test this hypothesis, histone methylation marks and LSD1 recruitment at the HIV-1 promoter were monitored in the latently infected U1 cell line after activation of viral gene transcription. As shown in Figure 2B, PMA (phorbol-12-myristate 13-acetate) treatment induced a release of the endogenous LSD1 from the viral promoter (column 2). Moreover, this phenomenon was associated with decreased trimethylation levels of H3K4 and H3K9 (columns 3 and 4 from Figure 2B, respectively). As expected, the release of the endogenous LSD1 following PMA treatment of the latently infected U1 cell line was associated with an increase of the global histone H3 acetylation level and with an increased RNA pol II recruitment to the HIV-1 promoter (Figure 2B columns 6 and 7). To verify whether LSD1 is specifically located at the promoter region of the HIV-1 genome, we performed several ChIP experiments with additional sets of primers hybridizing in adjacent regions of the viral genome. As shown in Figure 2C, LSD1 was only associated to the proximal promoter region (columns 1 and 2) and not with adjacent regions such as intragenic Gag or Vpr regions (columns 3 and 4). Moreover, the epigenetic marks associated with the loss of LSD1 we described above were also observed with other LSD1 regulated genes since we observed the same events with the LSD1-regulated gene CEBP alpha (Figure 2D, columns 5, 6 and 7), as previously described (35). In accordance with the literature (36), we showed that knock-down of LSD1 is correlated with an increase of H3K4me3 in the promoter region of LSD1 regulated genes such as SCN1A, SCN3A (data not shown) and SCN2A2 (Figure 2D columns 2, 3 and 4), suggesting that these LSD1-sensitive genes may be regulated by molecular mechanisms linked to the previously described enzymatic activity of LSD1. As a control, we showed that LSD1 is not associated to the promoter of the LSD1-insensitive gene GAPDH (Figure 2D). In addition, H3K4 trimethylation level at the GAPDH promoter was not sensitive to the modulation of LSD1 expression.

Taken together, these data suggest that LSD1 is recruited to the HIV-1 promoter and thereby represses its transcriptional activity. However, we were unable to show that this repression was linked to its previously characterized H3K4 or H3K9 demethylase activities.

LSD1 represses HIV-1 LTR-driven gene expression through the Sp1-binding sites of HIV-1 promoter

In order to identify the LTR region allowing the LSD1-mediated repression of HIV-1 transcription in microglial cells, we performed transient transfection experiments with 5′ truncations or mutations of the LTR-Luciferase

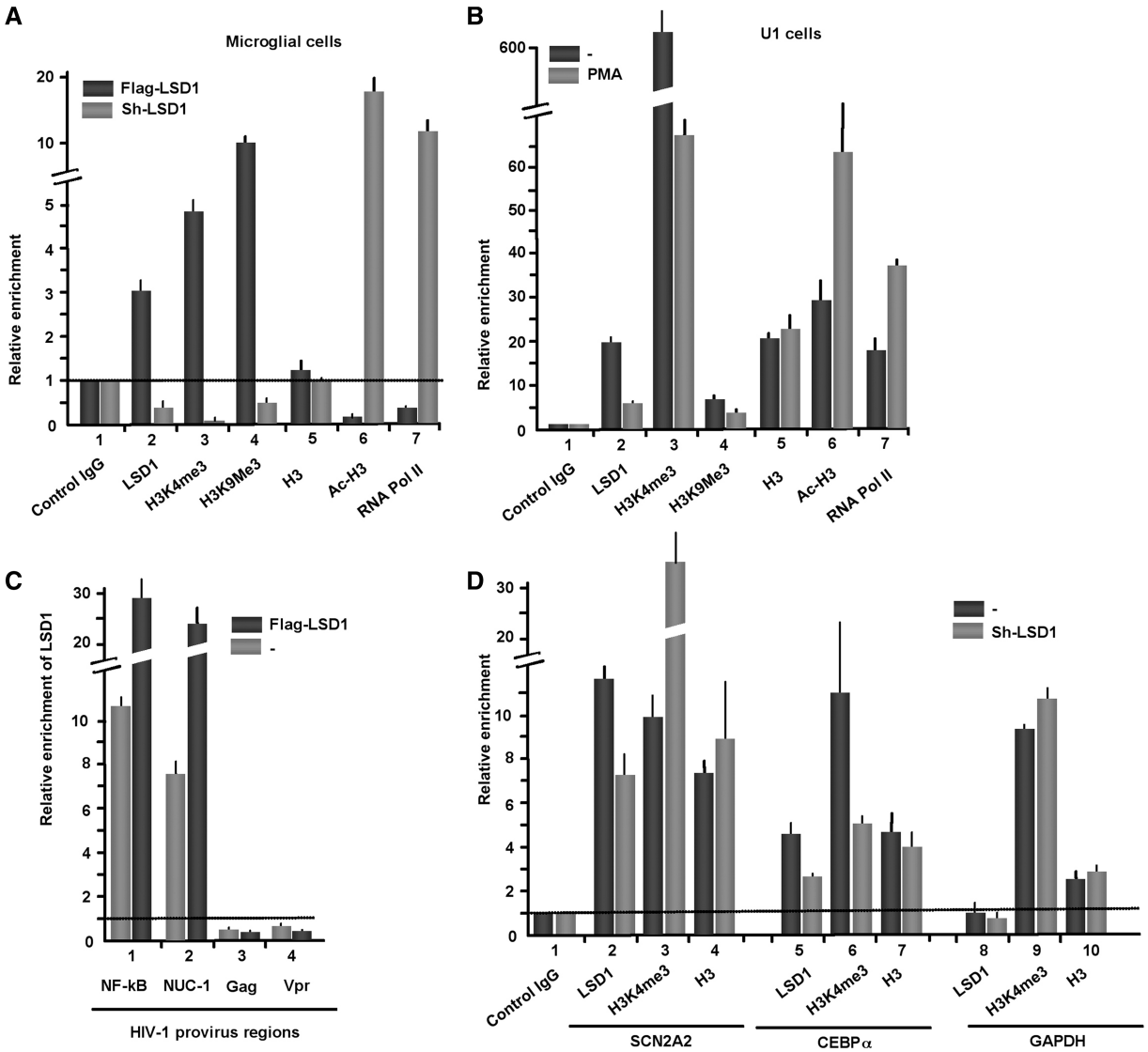

Figure 2. LSD1 association with the HIV-1 proximal promoter induces local trimethylation of histone 3 lysines 4 and 9. (**A**) ChIP experiments were performed on microglial cells transfected with the pNL-4.3 provirus in the presence of the pFLAG-LSD1, the pshRNA-LSD1 or the respective pcDNA3-FLAG and pshRNA-control vectors. Cells were subjected to ChIP assays with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR targeting the Nuc-1 region. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of LSD1 over-expression or depletion were expressed relative to the value obtained with the pcDNA3-FLAG or the pshRNA-control vectors, respectively. (**B**) Mock-treated and PMA-treated U1 cells were subjected to ChIP experiments with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR targeting the Nuc-1 region. The amounts of immunoprecipitated material were normalized to the input DNA and presented relative to the non specific IgG precipitation. (**C**) pcDNA3 and pFLAG-LSD1 transfected microglial cells were subjected to ChIP experiments with the anti-LSD1 antibody. Specific enrichment of the NF-kB and the Nuc-1 regions of the promoter and the Gag and Vpr intragenic regions are presented relative to the non specific enrichment obtained with the control IgG setted at 1. (**D**) pshRNA-control and pshRNA-LSD1 transfected microglial cells were subjected to ChIP experiments with the indicated antibodies. Specific enrichment of the SCN2A2, CEBPα and GAPDH promoters are indicated relative to the control IgG.

vector in the context of ectopic LSD1 over-expression or endogenous LSD1 knock-down. While LSD1 over-expression repressed the luciferase expression of the full-length and the proximal LTR constructs, knocking-down LSD1 stimulated these transcriptional activities (Figure 3A lanes 1 and 2). Interestingly, mutation of the Sp1-binding sites abrogated LSD1-mediated repression, suggesting the involvement of this LTR region in LSD1 recruitment (Figure 3A lane 3). To establish whether the endogenous LSD1 associates with the viral promoter via the Sp1-binding sites, we performed ChIP experiments with the wild-type LTR-Luc (wild-type 1–789) and the Sp1-binding sites-mutated vectors. As shown in Figure 3B (column 3), mutation of the Sp1-binding sites abolished LSD1 recruitment at the vial promoter. Interestingly, we observed that the epigenetic mark H3K4me3 is preferentially detected in the wild-type LTR (pLTR-Luc wt) (Figure 3B column 4). Since it has been shown that

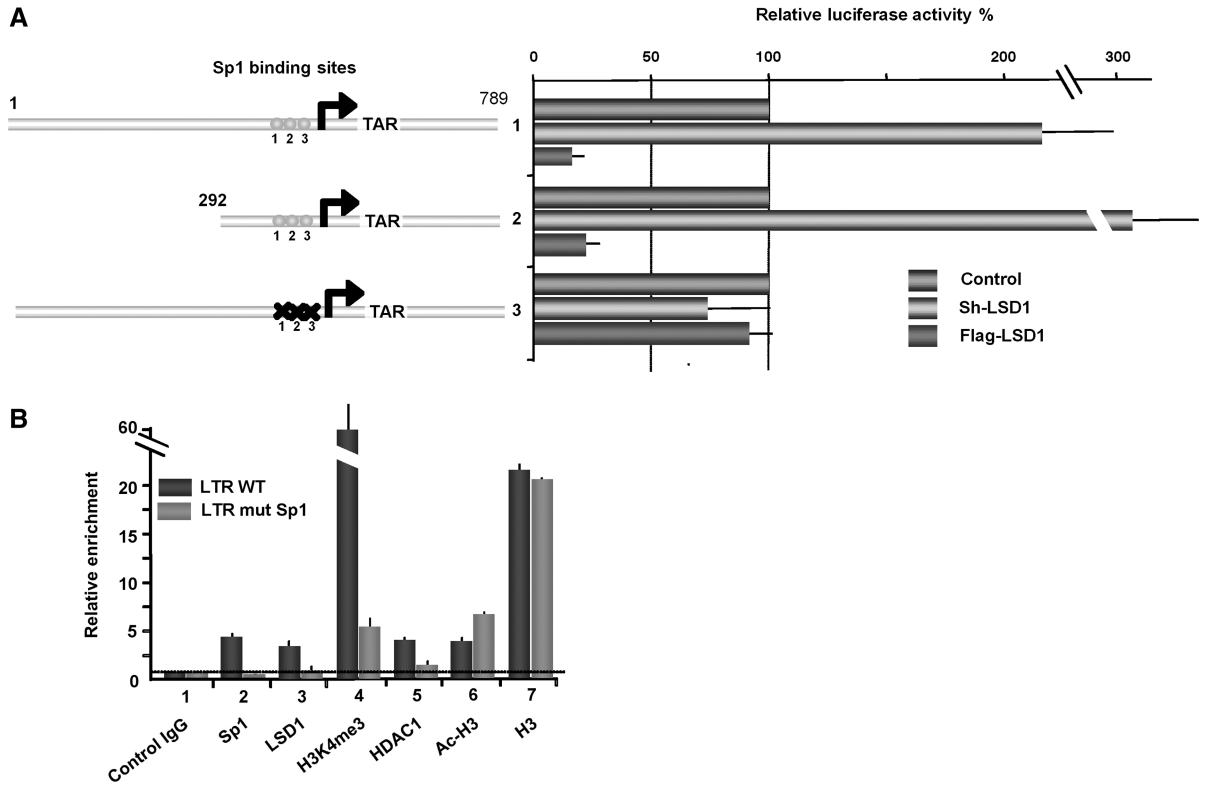

Figure 3. LSD1-mediated repression of HIV-1 gene transcription and replication requires HIV-1 proximal promoter Sp1-binding sites. (**A**) Microglial cells were transfected with 1 µg of the pLTR-LUC (1–789), pLTR-LUC (292789) or the pLTR-LUC (1–789) mut Sp1 and 1.5 µg of the pFLAG-LSD1 or pshRNA-LSD1 vectors. pcDNA3-FLAG and pshRNA-control plasmids were used to normalize the transfected DNA amounts. Two days post-transfection, Luciferase activities were measured and the results are expressed relative to the control vectors. (**B**) HEK 293 T cells were transfected with the pLTR-LUC (1–789) or with the pLTR-LUC (1–789) mut Sp1 vector 48 h before being subjected to ChIP experiments with the indicated antibodies. Input and immunoprecipitated DNAs were quantified by real-time PCR using primers targeting the Sp1-binding sites region of the HIV-1 promoter. The amounts of immunoprecipitated material were normalized to the input DNA and results are presented relative to the non-specific control IgG.

Sp1 recruits HDAC1 (37), we compared the association of HDAC1 on the WT and the Sp1-mutated viral promoter. As shown in Figure 3B, the global recruitment of HDAC1 to the HIV-1 promoter was mostly abrogated by the mutation of the Sp1-binding sites (column 5). Interestingly, this was correlated with a small increase of the H3 acetylation level (Figure 3B column 6) and a strong decrease of the H3K4 trimethylation level (Figure 3B column 4). In a control experiment, we verified that the same amounts of histone H3 was found on both constructs (Figure 3B column 7).

LSD1 cooperates with CTIP2 to repress HIV-1 replication and transcription

We have previously shown that CTIP2 represses HIV-1 gene transcription in microglial cells (13). We therefore hypothesized that CTIP2 and LSD1 could cooperate to repress HIV-1 replication and transcription. As shown in Figure 4A, the knock-down of both factors synergistically activated HIV-1 replication (30-fold activation with the double knock-down compared to the 5-fold and 10-fold activations observed with the LSD1 and CTIP2 single knock-downs, respectively).

The transcriptional impacts of LSD1 and CTIP2 knock-downs were then assessed in the presence or absence of Tat. Single knock-down of CTIP2 stimulated transcription in the absence and presence of Tat (Figure 4B columns 2 and 3 and C columns 3 and 4). However, combination of LSD1 and CTIP2 knock-downs further increased HIV-1 gene transcription in the absence or presence of Tat (Figure 4B column 4 and 4 C column 5). These results strongly suggest a functional cooperation between LSD1 and CTIP2 in the repression of HIV-1 transcription in a chromatinized promoter. As controls, knock-down and over-expression efficiencies were verified by western blot (Figure 4D).

LSD1 interacts with CTIP2 and co-localizes with Tat and CTIP2 in the nucleus

Our data strongly suggest a functional cooperation between LSD1 and CTIP2. We therefore investigated whether these proteins could interact physically. To this end, we performed FLAG-targeted immunoprecipitation experiments with nuclear extracts from cells expressing FLAG-LSD1 or FLAG-CTIP2 proteins. As shown in Figure 5A, FLAG-CTIP2 and FLAG-LSD1 co-immunoprecipitated with endogenous LSD1 and CTIP2 proteins,

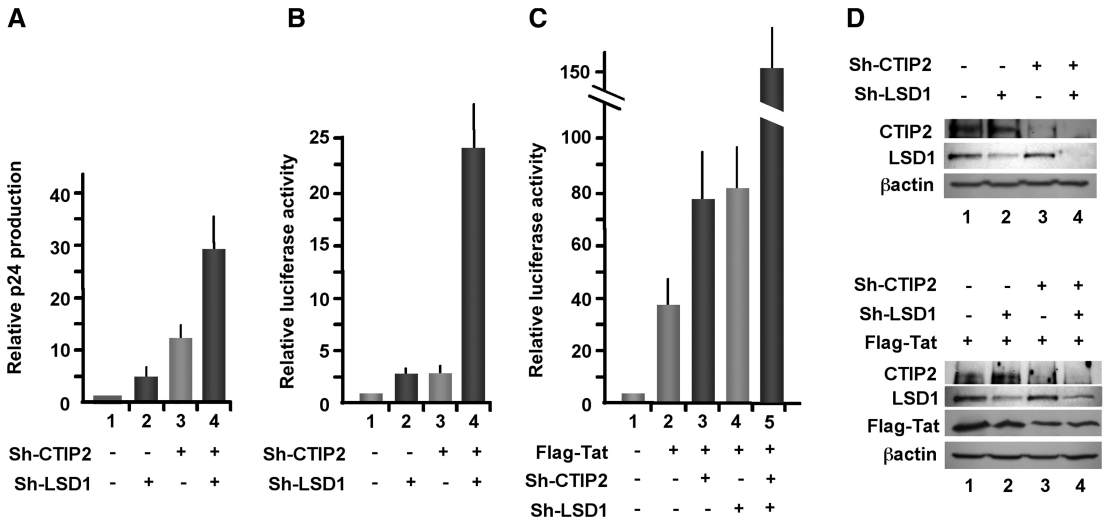

Figure 4. LSD1 cooperates with CTIP2 to repress HIV-1 gene transcription and viral replication. (**A**) Microglial cells were transfected with pNL-4.3 and the indicated plasmids (columns 2 to 4) or the pshRNA-control vector (column 1). Culture supernatants were analysed for p24 contents 48 h post-transfection. (**B** and **C**) Microglial cells were transfected with the episomal LTR-LUC and the indicated plasmids or the pshRNA-control vector. Luciferase activities were measured 2 days post-transfection and expressed relative to the value obtained with the episomal LTR-LUC and the control vectors (columns 1). DNA quantities were normalized with the pshRNA-control vector. (**D**) The knock-down efficiency of sh-RNA constructs was controlled by western blot analysis. The control columns 1 of the panels correspond to extracts from cells transfected with the pshRNA-control vector.

respectively, arguing for a physical interaction between these two proteins. We next investigated whether LSD1 co-localizes with Tat as previously shown for CTIP2 (30). Cells transfected with a RFP-CTIP2 expressing vector in the presence or not of GFP-Tat were examined for endogenous LSD1 localization using confocal microscopy. Endogenous LSD1 expression was observed in both the cytoplasm and the nucleus (Figure 5B, pictures 4 and 6). As previously described (13), nuclear expression of CTIP2 harboured ball-like structures (Figure 5B, pictures 7 and 9). As shown in Figure 5C (pictures 5–8), LSD1 and CTIP2 co-localized in the CTIP2-induced nuclear structures (30), suggesting that CTIP2 relocated LSD1 into these structures. Interestingly, GFP-Tat expression relocated LSD1 from the cytoplasm to the nucleus (Figure 5C, pictures 1–4). Finally, observations of the concomitant expressions of RFP-CTIP2 and GFP-Tat revealed co-localization of both proteins with LSD1 in the nucleus (Figure 5D). Staining of genomic DNA are presented in Figure 5B. Altogether, these results support that CTIP2 and LSD1 interact physically and that LSD1 is re-localized by CTIP2 and Tat in dense sub-nuclear structures.

LSD1 is required for CTIP2 recruitment at the HIV-1 proximal promoter

We next asked whether LSD1 is required for CTIP2 recruitment to the HIV-1 promoter. To address this question, we performed additional ChIP experiments in the LSD1 over-expression or LSD1 knock-down contexts. As shown in Figure 6A, over-expression of LSD1 was associated with an increase of endogenous CTIP2 recruitment to the viral promoter. As expected, LSD1 knock-down decreased CTIP2 association with the viral promoter (Figure 6A). To further study LSD1 and CTIP2 recruitment at the HIV-1 promoter, we performed ChIP experiments with HIV-1 infected microglial cells expressing (control) or not CTIP2 (shCTIP2) (Figure 6B). As a control, we checked that CTIP2 is less recruited onto the HIV-1 proximal promoter in the infected shCTIP2 microglial cell line (Figure 6B column 2 compared to column 1) compared to the control cell line. Unexpectedly, knocking-down CTIP2 slightly increased LSD1 recruitment to the LTR (Figure 6B column 3). Moreover, this recruitment was correlated with an increased H3K4 trimethylation (Figure 6B column 4). These results suggest that LSD1 is required for CTIP2 recruitment to the HIV-1 proximal promoter.

LSD1-mediated repression and H3K4 trimethylation are associated with the recruitment of hSET1 and WDR5 to the HIV-1 proximal promoter

The epigenetic mark H3K4me3 has been shown to be associated with LSD1 recruitment (20,21). This association results from the interaction of LSD1 with a methyltransferase complex containing WDR5 and hSET1 (20,21). ChIP experiments performed with cells overexpressing LSD1 confirmed an increased recruitment of hSET1 and WDR5, two members of the hCOMPASS complex (Figure 7A, blue columns 4 and 5) to the HIV-1 promoter, together with an increased H3K4 trimethylation. Inversely, knocking-down endogenous LSD1 decreased hSET1 and WDR5 association to the viral promoter and H3K4 trimethylation (Figure 7A pink columns). ChIP experiments performed with the wt-LTR-Luc or the Sp1-mutated LTR-Luc reporter constructs further confirmed that hSET1, WDR5 and LSD1 are recruited concomitantly to the proximal Sp1-binding

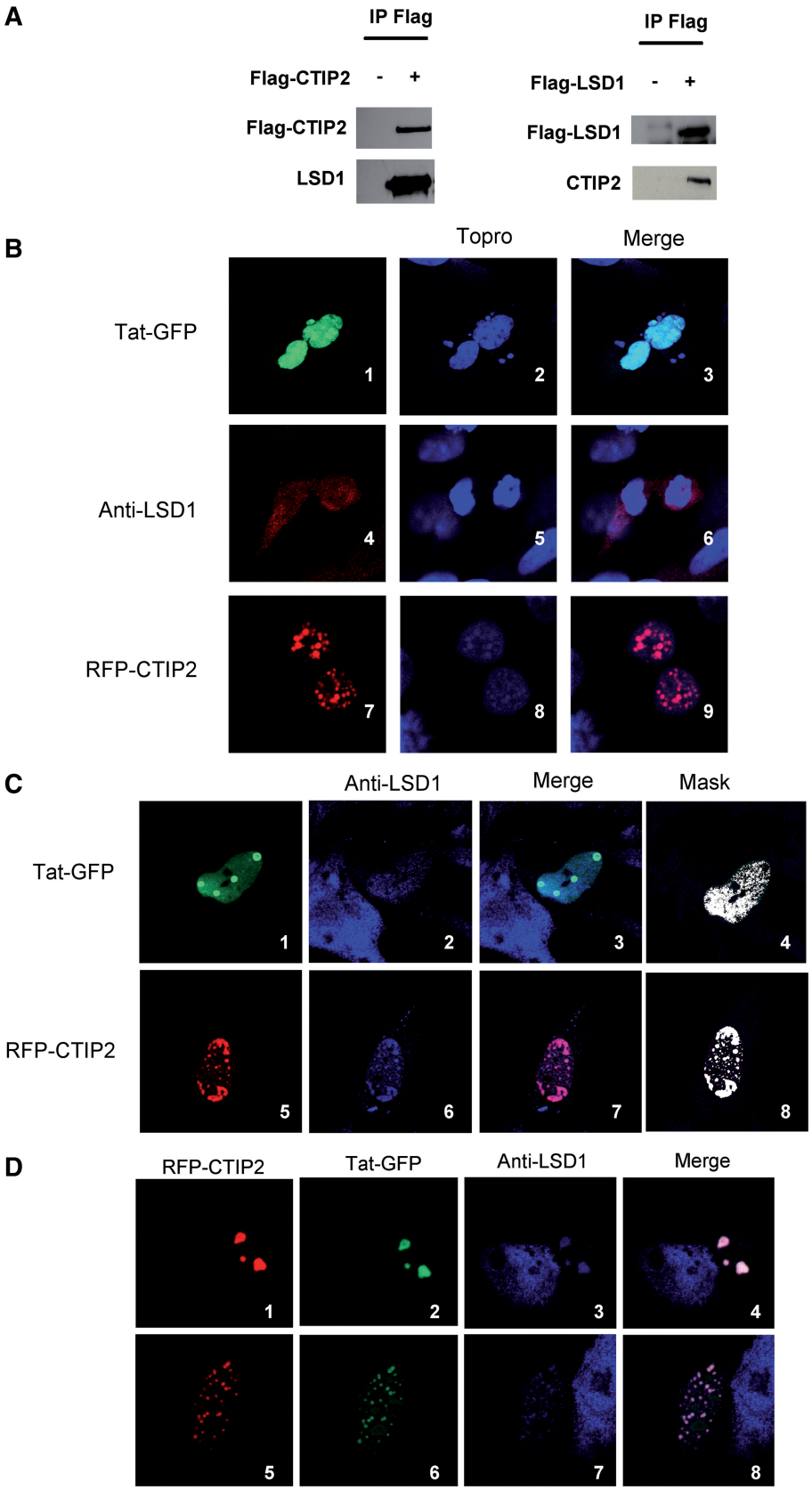

Figure 5. LSD1 associates with CTIP2 and co-localizes with CTIP2 and Tat within nuclear structures. (**A**) HEK 293 T cells were transfected with the pFLAG-CTIP2, the pFLAG-LSD1 expression vectors or the control pCDNA3-FLAG vector. Complexes immunoprecipitated with the anti-FLAG antibody were immunodetected for the presence of FLAG-CTIP2, FLAG-LSD1, endogenous LSD1 and CTIP2 proteins by western blot as indicated. (**B–D**) Microglial cells were transfected with pTat-GFP or/and pRFP-CTIP2 as indicated and accessed for endogenous LSD1 immunodetection with primary anti-LSD1 antibodies. The primary immunocomplexes were revealed by CY3- or CY5-labeled secondary antibodies. Mask columns show the co-localized staining.

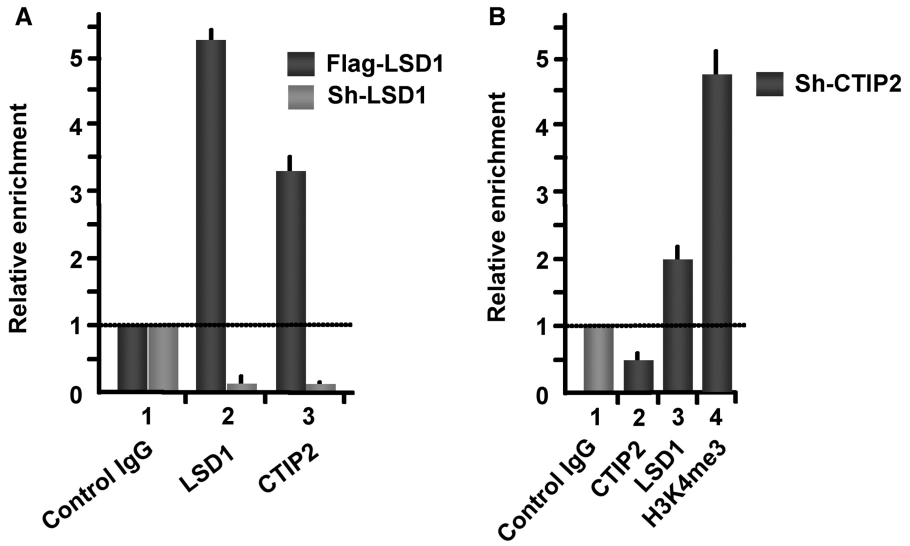

Figure 6. CTIP2 recruitment on the HIV-1 proximal promoter requires LSD1. (**A**) ChIP experiments were performed on HEK 293 T cells transfected with the HIV-1 LTR LUC episomal vector in the presence of the pFLAG-LSD1, the pshRNA-LSD1 or the respective pcDNA3-FLAG and pshRNA-control vectors. Cells were subjected to ChIP assays with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR targeting the Sp1-binding sites. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of LSD1 over-expression or LSD1 depletion were expressed relative to the value obtained with the pcDNA3-FLAG and the pshRNA-control vectors, respectively. Results were expressed relative to the value obtained with the episomal LTR-LUC plasmid co-transfected with the pcDNA3-FLAG or the pshRNA-control vectors. (**B**) Control and CTIP2 knocked-down microglial cells were infected with the VSV-pseudotyped pNL-4.3-Env virus 24 h before being subjected to ChIP experiments with the indicated antibodies. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of CTIP2 depletion were expressed relative to the value obtained with the control cells. Specific enrichments at the HIV-1 proximal promoter were quantified by real-time PCR targeting the LTR-Sp1-binding sites region.

sites of the viral promoter. Indeed, the abrogated association of LSD1 to the Sp1-mutated LTR (Figure 7B lane 2 column versus column) correlated with a reduced recruitment of both hSET1 and WDR5 (Figure 7B, columns 3 and 4). From these results, we hypothesized that HIV-1 reactivation in microglial cells could be associated with a release of LSD1 and an alongside reduced recruitment of hSET1 and WDR5 to the HIV-1 promoter. In agreement with these results, PMA treatment released LSD1, WDR5 and hSET1 from the viral promoter of HIV-1 infected cells (Figure 7C, columns 2, 4 and 5) and decreased H3K4 trimethylation (Figure 7C column 3). Taken together, our data suggest that LSD1-associated increase of H3K4 trimethylation at the HIV-1 proximal promoter region might be linked to hSET1 and WDR5 recruitment.

DISCUSSION

The introduction of HAART in 1996 has raised hopes for curing patients infected with HIV-1. Unfortunately, long-term suppression of HIV-1 replication has unveiled the existence of latent HIV-1 reservoirs such as resting $CD4^+$ T cells and monocytes/macrophages (25,38). Microglial cells, the CNS-resident macrophages, are the brain major targets for HIV-1 and constitute latently infected cells (39). Understanding the molecular mechanisms of establishment, maintenance and reactivation of HIV-1 latency in microglial cells is therefore crucial for efficient therapeutic intervention (26,27). In recent papers, we reported that CTIP2 inhibits HIV-1 replication and transcription in microglial cells (29,30) by recruiting a chromatin-modifying complex which contains histone deacetylases (HDAC1 and HDAC2) and a histone methyltranferase (SUV39H1) (13). A better comprehension of the molecular mechanisms involved in establishment and maintenance of HIV-1 latency would be achieved by the identification of additional factors able to induce heterochromatin formation at the viral promoter (26,31). Since histone and DNA methylations have been implicated in the silencing of the integrated provirus (12,13,40,41), we further investigated the influence of specific demethylase enzymes. LSD1, a demethylase first identified by Shi *et al.* (14) as a transcriptional repressor, constituted therefore a potential candidate to play a role in HIV-1 silencing. In this report, we first showed that LSD1 represses HIV-1 expression by inhibiting the transcription step of the viral life cycle. ChIP experiments revealed that LSD1 is recruited to the HIV-1 proximal promoter. In addition, the repressive epigenetic mark H3K9me3 was linked to LSD1 recruitment at the viral promoter. Since LSD1 was initially characterized as a repressor associated with demethylation of H3K4 (42), we focused our investigation on this point. Surprisingly, we observed an increased H3K4 trimethylation level in the HIV-1 proximal promoter region upon LSD1 recruitment. Such a pattern has previously been described in a previous work in the context of the HSV promoter by Liang *et al.* (21). However, these authors have further shown that this H3K4 trimethylation correlated with a demethylation of H3K9 and with transcriptional activation (21). Here, we demonstrate for the first time that LSD1-induced

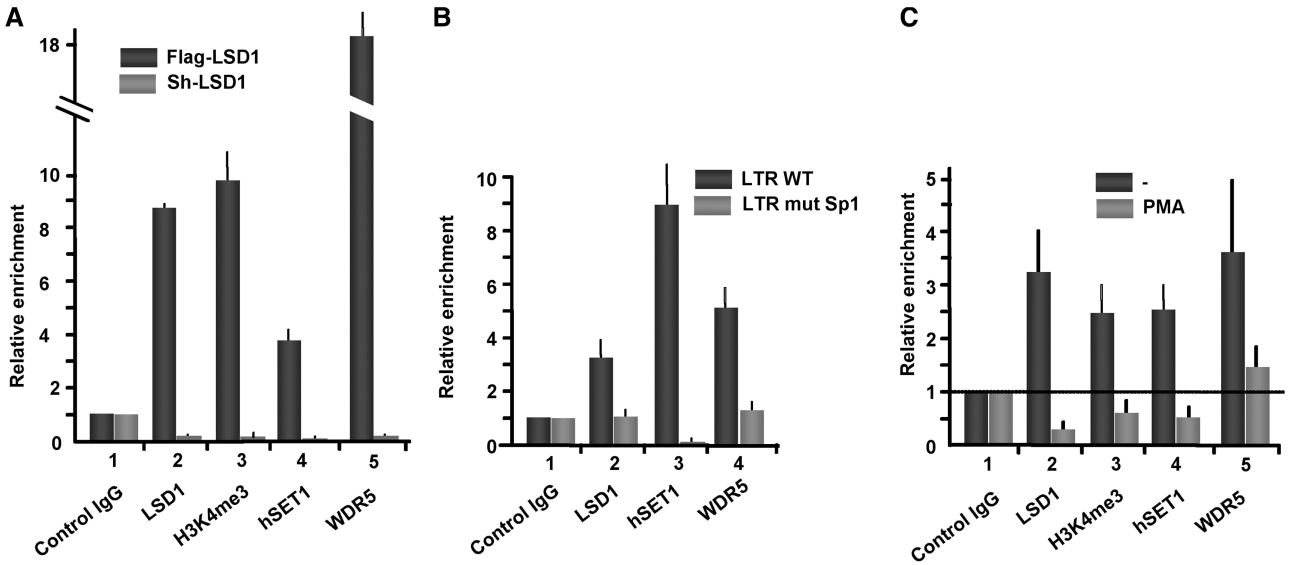

Figure 7. LSD1 favours hSET1 and WDR5 recruitment at the HIV-1 proximal promoter. (**A**) ChIP experiments were performed on HEK 293 T cells transfected with the HIV-1 LTR-LUC episomal vector in the presence the pFLAG-LSD1, the pshRNA-LSD1 or the respective pcDNA3-FLAG and pshRNA-control vectors. Cells were subjected to ChIP assays with the indicated antibodies. Specific enrichments were calculated relative to the control IgG and relative enrichments in the context of LSD1 over-expression or depletion were expressed relative to the value obtained with the pcDNA3-FLAG or the pshRNA-control vectors, respectively. Specific enrichments in the HIV-1 proximal promoter region were quantified by real-time PCR. (**B**) HEK 293 T cells were transfected with the pLTR-LUC (1–789) or with the pLTR-LUC (1–789) mut Sp1 vector 48 h before being subjected to ChIP experiments with the indicated antibodies. Input and immunoprecipitated DNAs were quantified by real-time PCR using primers targeting the Sp1-binding sites region of the HIV-1 promoter. The amounts of immunoprecipitated material were normalized to the input DNA and presented relative to the non specific control IgG. (**C**) Microglial cells were infected with the VSV-pseudotyped pNL4.3-Env virus 24 h before being subjected to ChIP experiments with the indicated antibodies. Specific enrichments in the HIV-1 proximal promoter were quantified by real-time PCR. Enrichments are presented relative to the non specific IgG values set at 1.

transcriptional repression can be associated with both H3K4me3 and H3K9me3 epigenetic marks. We further show that these epigenetic marks are linked to LSD1 recruitment at the HIV-1 proximal promoter through the Sp1-binding sites as previously demonstrated for CTIP2 (13). Our results are in agreement with the model presented by the David Margolis group who demonstrated that HDAC1 is released from the HIV-1 promoter upon mutation of the Sp1-binding sites (37). Indeed, we demonstrated that LSD1 and HDAC1 are associated with the wild-type but not with the Sp1 mutated promoter. New functional and biochemical investigations will be needed to determine if these enzymes compete or cooperate for their binding to the HIV-1 promoter. However, it is now clear that both enzymes are involved in the silencing of HIV-1 gene transcription.

We next evaluated whether two epigenetic marks, H3K9me3 and H3K4me3, could result from LSD1 recruitment onto the HIV-1 promoter. Regarding H3K9me3, we speculated that LSD1 could cooperate with CTIP2 as we had previously shown that this factor recruits SUV39H1, enzymes which specifically methylates H3K9 (13). In agreement with this hypothesis, we showed here that LSD1 cooperates functionally with CTIP2 to repress HIV-1 replication and transcription in a synergistic manner. We further demonstrated that this cooperation correlates with a physical interaction between CTIP2 and LSD1. ChIP assays showed that LSD1 and CTIP2 interact physically, and confocal microscopy experiments suggested an *in vivo* interaction between these two proteins, which was found to occur in previously characterized CTIP2-induced nuclear structures (30). In addition, the transactivator Tat relocated LSD1 in the nucleus. Since we believe that these ball-like nuclear structures reflect a heterochromatin environment, the co-localization of LSD1 with both CTIP2 and Tat strongly suggests that LSD1 is involved in promoting local heterochromatin environment to repress HIV-1 gene transcription in microglial cells. Moreover, LSD1 seems to have a more critical role in promoting HIV-1 silencing than CTIP2. Indeed, we showed that LSD1 is required for CTIP2 recruitment onto the HIV-1 proximal promoter whereas the reverse is not observed.

Mechanisms underlying LSD1-mediated increase of H3K4 trimethylation might rather rely on LSD1 ability to anchor other factors at the promoter than to its own enzymatic activity. Indeed, LSD1 is also known to be associated with the recruitment of hSET1 and WDR5, two members of the hCOMPASS methyltransferase complex, which is believed to induce the trimethylation of H3K4 (43–46). In this context, LSD1, through its interaction with HCF-1, recruits this methyltransferase complex containing WDR5 and hSET1 to the HSV promoter (21). Here, we showed that LSD1 favours the recruitment of such a complex to the HIV-1 promoter. H3K4 trimethylation was associated with the recruitment of LSD1, hSET1 and WDR5 at the Sp1-binding sites of the HIV-1 LTR. Moreover, reactivation of HIV-1

proviruses correlated with the release of LSD1, hSET1 and WDR5 from the viral promoter and with a reduced H3K4 trimethylation. All together, our results strongly suggest that LSD1 is involved in the establishment and the maintenance of HIV-1 latency in microglial cells by favouring a local heterochromatin structure.

Association of both H3K4me3 and H3K9me3 epigenetic marks with LSD1 recruitment may thus constitute a new level of eukaryotic gene regulation. These observations are consistent with the discovery that H3K4 methylation at certain chromatin loci may prevent gene expression (11). Interestingly, such a gene repression linked to H3K4me3 has been proposed to prevent the expression of cryptic promoters (5,11). This is strengthened by the findings that HIV-1 preferentially integrates in active genes and therefore could be considered as a cryptic gene. Additional mechanisms including transcriptional interference are believed to prevent expression of such cryptic promoters (47). It could be argued that H3K4me3 is already present on the HIV-1 promoter upon integration into the host cell genome as it integrates into active genes enriched with euchromatic histone modifications such as H3K4 methylation and histones acetylation (48). The subsequent silencing characterized by H3K9me3 would then occur through LSD1-mediated recruitment of the CTIP2-HDAC-SUV39H1 complex. However, the fact that both H3K4me3 and H3K9me3 were lost following reactivation in association with the release of LSD1 from the HIV-1 promoter argues that, at least in the context of HIV-1 promoter, these two epigenetic marks are associated with transcriptional repression and favour the establishment and the maintenance of latency.

The exact mechanisms underlying LSD1 function in HIV-1 repression are complex and far to be elucidated. Although we showed in this report that LSD1 interacts physically and cooperates functionally with CTIP2, it remains to be determined whether all the components recruited to the HIV-1 promoter are in the same complex or whether they interact with LSD1 independently. Even if the exact mechanisms by which LSD1 promotes a local heterochromatin structure remain unknown, LSD1 constitutes a new target for potential therapeutic strategies aiming at purging the HIV-1 latent reservoirs.

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